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(54) Title: LABELING OF NUCLEIC ACIDS WITH FLUORESCENT MARKERS (57) Abstract The present invention relates to DNA markers and, particularly, nucleic acid labeling techniques. More specifically, this invention contemplates a protocol which permits the covalent introduction of single or multiple fluorescent markers or other probes into DNA fragments and oligodeoxynucleotides. The instant technique, particularly employing multiple fluorescent markers, allows high sensitivity detection of nucleic acids (without the use of sophisticated detection devices) in the low femtomolar (10^{-15} mol) range and additionally permits the placement of markers and probes at specific locations within the macromolecule. The present invention can be used with high detection sensitivity for DNA sequencing and hybridization procedures including a host of diagnostic and therapeutic procedures. The present technique can be employed as a tool for the study of nucleic acid dynamics through recognition and evaluation of fluorescence energy transfer and electron spin resonance, and the study of structure, conformation and dynamics of biopolymers. Specific labeling procedures allow the introduction of a probe or other entity for the location of desired sequences or the delivery of the probe to a specific sequence. This process is fundamental to the emerging fields of DNA diagnostics and therapeutics.		

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LABELING OF NUCLEIC ACIDS WITH FLUORESCENT MARKERS

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FIELD OF THE INVENTION

The present invention relates to DNA markers and, particularly, nucleic acid labeling techniques. More specifically, this invention contemplates a protocol which permits the covalent introduction of single or multiple fluorescent markers or other probes such as spin labels and drug analogues into DNA fragments and oligodeoxynucleotides. The instant technique, particularly employing multiple fluorescent markers, allows high sensitivity detection of nucleic acids (without the use of sophisticated detection devices) in the low femtomolar (10^{-15} moles) range and additionally permits the placement of markers and probes at specific locations within the macromolecule. The present invention can be used with high detection sensitivity for DNA sequencing and hybridization procedures including a host of diagnostic and therapeutic procedures. The present technique can also be employed as a tool for the study of nucleic acid dynamics through recognition and evaluation of fluorescence energy transfer and electron spin resonance, and the study of structure, conformation and dynamics of biopolymers. Specific labeling procedures allow the introduction of a probe or other entity for the location of desired sequences or the delivery of the probe to a specific sequence. This process is fundamental to the emerging fields of DNA diagnostics and therapeutics.

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BACKGROUND OF THE INVENTION

The determination of the presence of nucleic acid fragments has typically relied on the use of radioisotopic labeling techniques. The enormous utility of these techniques has largely been a function of the high sensitivity associated with their detection. Such

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1 sensitivity has allowed the location of quantities of
material in amounts in the low femtomolar range (10^{-15}
moles). However, the use of radioisotopes is rendered less
than ideal by the associated problems of safety and disposal.

5 Fluorescent rather than radioisotopic labeling
procedures are an attractive option which avoids these
liabilities, but fluorescent labeling procedures have
previously been compromised by their greatly reduced
sensitivity. Fluorescent dyes as well as spin labels are
also useful in many aspects of biophysics since the
10 properties of a given marker can vary substantially with
changes in the immediate microenvironment. Such probes can
be useful for the study of structure, conformation and
dynamics in biopolymers providing that they can easily be
placed at specific locations within the desired
15 macromolecule.

In order for fluorescent labeling procedures to
compete effectively with and replace radioisotopic labeling
techniques for the detection of macromolecules during various
biochemical assays, the fluorescent labeling must result in
20 high detection sensitivity, rapid and simple procedures for
the introduction of the fluorescent marker to the
macromolecule of interest must be available, and the results
must be reproducible. By meeting these criteria and with the
additional advantage of reduced health hazards, fluorescent
25 labeling techniques could then replace the use of
radioisotopes in a number of biochemical assays.

Intercalative dyes such as ethidium bromide
generally meet these criteria and in many cases have
completely replaced radioisotopic labeling procedures for the
30 detection of double stranded DNA. However, a number of
assays, including DNA sequencing and hybridization

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1 techniques, cannot benefit from intercalative fluorescent
labeling. These procedures require that the fluorescent
marker be covalently bound to the nucleic acid, and the
intercalative dye is unable to meet this requirement.

5 All prior studies for the covalent attachment of
fluorescent markers to nucleic acids, until the present
invention, suffered from at least one of two disadvantages.
First, attachment of only a single label to the nucleic acid
(usually at one of the termini) severely compromised its
10 detection. Secondly, although multiple labeling techniques
can enhance detection sensitivity, they have generally
required the time-consuming synthesis of a modified
nucleoside derivative containing a fluorophore or one which
can be modified with a fluorophore. In addition to
fluorophores, the use of biotin as a non-radioactive labeling
15 technique has also been considered.

The use of single labels, usually at the terminus
of the nucleic acid fragment, is the conventional state of
the art primarily because it is chemically and enzymatically
easier to exploit modification reactions at a nucleic acid
20 terminus rather than at a specific point in the internal
regions of the sequence. Additionally, the placement of the
marker at one of these termini also removes the marker from
the "site of action" when monitoring protein binding or any
process where an essentially native DNA sequence is required.
25 It has commonly been difficult to detect fragments containing
a single fluorescent marker with the high sensitivity
available with a radioisotopic label. Although problematic,
labeling with a single fluorophore has been accomplished
using both chemical and enzymatic techniques. DNA sequencing
30 has been attempted using such labeling techniques but
requires sophisticated electronic detection, and then only
has evidenced limited success.

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Several methods have been reported for the
1 incorporation of multiple labels into nucleic acids. Most of
these rely on an enzymatic polymerization reaction in order
to introduce a modified nucleoside carrying the desired label
or one which can be easily modified with the fluorescent
5 marker at numerous positions. Base-specific reactions have
also been employed, such as modification of guanine residues
with N-acetoxy-2-acetylaminofluorene followed by detection
with tetramethylrhodamine-labeled antibodies raised against
the modifying reagent. Multiple labeling techniques have
10 commonly resulted in enhanced detection sensitivity with
respect to single labels and have been reasonably
reproducible. However, these techniques have previously not
been simple or rapid to employ. The modified nucleoside has
previously only been obtained by time-consuming chemical
15 syntheses.

Another prior approach involves the use of biotin
labeling. While biotin itself is not a fluorescent
chromophore, biotin labeling when combined with
immunochemical, histochemical or affinity detection systems
20 provides another alternative to radioisotopic labeling of
nucleic acids. Biotin-labeled nucleic acids have been used
in hybridization studies, gene mapping studies employing
electron microscopy and gene enrichment in cesium chloride
gradients. Biotin labeling has been typically approached in
25 conceptually the same manner as fluorescent labeling
techniques in which either a single label at the nucleic acid
terminus or multiple labels requiring the synthesis of a
biotin labeled dNTP derivative are employed. Generally, each
of the existing techniques suffers from the requirements of
30 arduous chemical synthesis and/or limited detectability.

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Conventional techniques when applied to DNA sequencing procedures add additional complications since the DNA fragments prepared during sequencing techniques must be resolved by electrophoresis in a polyacrylamide gel matrix. Since electrophoresis procedures resolve nucleic acid fragments on the basis of size (or molecular weight), the addition of one or more fluorescent labels to the fragments prior to electrophoresis results in anomalous migration of the DNA within the gel and undue complications in the analysis of the sequence. The most desirable procedure for employing fluorescent labeling techniques in DNA sequencing and hybridization procedures would involve the incorporation of multiple labels into the nucleic acid or hybridization probe (to enhance detection sensitivity), before or after electrophoretic resolution of such fragments or before or after hybridization of the probe onto a nitrocellulose membrane ("pre-assay" or "post-assay" labeling). Multiple covalent labeling of nucleic acids with fluorophores in a "post-assay" manner has not been previously contemplated or described.

SUMMARY OF THE INVENTION

Accordingly, one object of the present invention is to provide an improved method for labeling nucleic acids.

Another object of this invention is to provide an improved method of fluorescently labeling nucleic acids.

A further object of the present invention is to provide new probes for use in DNA labeling and related techniques.

A still further object of this invention is to provide a new detection product which constitutes a phosphorothiolate diester covalently complexed with a nucleotidic residue, and which is also complexed with a detectable marker.

Another object of this invention is to provide
1 multiple sites, i.e., internally within the macromolecule,
for the attachment of fluorophores and other markers and/or
probes to the nucleic acid thereby enabling multiple labeling
techniques.

5 A further object of the present invention is to
selectively introduce fluorescent markers and other markers
and probes at specifically desired sites of the
macromolecule. These markers or reporter groups include
fluorophores, biotin, spin labels, drugs or their analogues,
10 hydrolytic reagents, chiral metal complexes and the like.

Another object of this invention is to selectively
introduce fluorescent markers and other probes after the
molecule of interest has been treated with any one of various
desired biochemical assays, i.e., in a "post-assay"
15 procedure.

Still another object of this invention is to
selectively introduce fluorescent markers and other probes
before the molecule of interest has been treated with any one
of various desired biochemical assays, i.e., in a
20 "pre-assay" procedure.

Yet another object of the present invention is to
provide an improved process for DNA sequencing, DNA
hybridization techniques and DNA diagnostics and DNA
therapeutics.

25 A still further other object of this invention is
to provide a new detection procedure which eliminates the use
of radioisotopes and the disadvantages associated with such
conventional methods.

30 These and other objects of the present invention
are achieved by providing a protocol which permits the
covalent introduction of single or multiple markers,
particularly fluorescent markers, and other probes into DNA
fragments and oligodeoxynucleotides at selective sites. More
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specifically, according to the present invention, nucleic
1 acids are labeled with markers such that, e.g., the
fluorescent marker or any other type of probe can be placed
into a specific location in the nucleic acid. By the
technique of the present invention, various sites for the
5 attachment of the desired probes or markers are generated by
employing phosphorothioate diesters in place of native
phosphodiesters which are chemically or enzymatically
introduced at the desired site within a nucleic acid and
subsequently marked with the desired reporter group. The
10 present methodology not only permits multiple labeling and
high sensitivity in a simple technique in the absence of
sophisticated detection devices, but also permits the
introduction of a particular probe or marker after
conventional biochemical assays, i.e., "post-assay." The
15 advantages of the novel detection products of this invention
also allow the labeling of DNA fragments in conventional DNA
sequencing or hybridization assays. Such assays further
permit a host of therapeutic procedures where a DNA
hybridization probe with attached phosphorothioate diester(s)
20 is employed in vivo or in vitro to locate a sequence within
genomic DNA and which is subsequently reacted with, e.g., a
label for detection or identification, a reactive molecule
for degradation, or other toxic therapeutic agents. The
novel product also allows study of the structure and dynamics
25 of nucleic acids as well as protein-nucleic acid complexes.
The novel product of the present invention includes a
nucleotidic residue covalently complexed with a
phosphorothioate diester and further complexed to a marker
enabling detection of the product.

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BRIEF DESCRIPTION OF THE DRAWINGS

1 Fig. 1 sets forth the structure of the
phosphorothioate triester composed of the nucleotidic residue
and phosphorothioate diester complexed with the bimane label
(bimane-Tp(S)T triester).

5 Fig. 2 is a graphic illustration of the stability
of the bimane-Tp(S)T triester at ambient temperature measured
during a total time period of 20 hours at pH values between
3-11.

10 Fig. 3 is a graphic depiction of an HPLC analysis
of the reaction mixture containing the octamer d[GC(s)CCGGGC]
(0.3 mM) and monobromobimane (3.0 mM) after reaction for
5 hours at ambient temperature.

15 Fig. 4 is a photographic reproduction of a
polyacrylamide gel (6%) illustrating "post-assay" labeling of
DNA fragments with monobromobimane.

 Fig. 4(A) represents an HpaII restriction
endonuclease digest of an M13mp18 DNA template, which has
been elongated with DNA polymerase I (E. coli) using dNTPs
and then treated with the endonuclease.

20 Fig. 4(B) represents an AvaI restriction
endonuclease digest of an M13mp19 DNA template, which was
elongated with DNA polymerase I (E. coli) using dNTPs and
then treated with endonuclease.

25 Fig. 5 represents phosphorothioate triester
oligodeoxynucleotides carrying (a) a PROXYL spin label. (b)
a derivative of the dihydropyrroloindole subunit of CC-1065,
(c) a sulfonamide-linked dansyl fluorophore, and (d) an
N-linked dansyl fluorophore.

DETAILED DESCRIPTION OF THE INVENTION

30 The present invention contemplates the selective
labeling of nucleic acids with fluorescent molecules and
other probes such as, for example, biotin, which are useful
in DNA sequencing and DNA hybridization assays. The present

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invention also contemplates other probes such as, for
1 example, spin labels which are useful in the analysis of
nucleic acid structure and dynamics. The convenient labeling
methodology of this invention further permits a broad range
of DNA therapeutic and diagnostic procedures and is
5 particularly characterized by the selective covalent
introduction of single or multiple markers and probes into
DNA fragments and oligodeoxynucleotides. The novel detection
product of this invention is characterized by a nucleotidic
residue covalently complexed with a phosphorothioate diester
10 which is mutually covalently complexed with a selected
marker. The probe is selectively introduced into a single
site of choice or into multiple sites as desired.

The present invention preferably employs a
phosphorothioate diester [for example, Tp(s)T,
15 phosphorothioate diester derivative of TpT (thymidyl(3'→5')
thymidine)] which is selectively incorporated into a DNA
fragment or oligodeoxynucleotide at any and each nucleotide
residue desired.

Specifically, the probe of the present invention, a
20 phosphorothioate diester derivative, is prepared by
introducing the phosphorothioate diester into the nucleic
acid fragment either enzymatically, e.g., according to the
method of Potter and Eckstein (Potter, B. and Eckstein, F.,
J. Biol. Chem., 259: 14243-14248, 1984), or chemically,
25 e.g., according to the method of Connolly, et al. (Connolly,
et al., Biochemistry, 23: 3443-3453, 1982).

The enzymatic technique of Potter and Eckstein
employs the desired dNTP α S 2'-deoxynucleoside-5'-0-
(1-thiotriphosphate), a suitable enzyme with polymerizing
30 characteristics such as DNA polymerase or reverse
transcriptase, a DNA template and a primer. The enzyme
employed, uses dNTP S as a substrate to synthesize nucleic
acids of varying chain length, and upon enzymatic reaction, a

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1 phosphorothioate diester is incorporated between two
nucleoside residues, along with the concurrent liberation of
pyrophosphate.

5 The phosphorothioate diester may be introduced
chemically into the nucleic acid by the method of Connolly,
et al. (or Stec, et al., J. Am. Chem. Soc., 106: 6077-6079).
This is generally a three step procedure. First, a phosphite
triester (nucleoside phosphite triester) is formed by
10 reacting a nucleoside phosphoramidite in the presence of a
weak acid such as tetrazole. Second, the phosphite triester
is oxidized in the presence of elemental sulfur (S_8), CS_2 and
lutidine, to form a phosphorothioate triester complex.
Third, in the presence of a base such as ammonia, the
phosphorothioate triester is hydrolyzed to the desired
phosphorothioate diester.

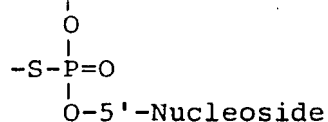
15 The selective introduction of the phosphorothioate
diester derivative into the DNA fragment or
oligodeoxynucleotide, is determined by the choice of
oxidation procedures at any given position. As explained
above, the phosphorothioate diester is obtained by oxidation
20 in the presence of S_8 , CS_2 and lutidine. The native
phosphate diester is obtained by oxidation of the phosphite
triester with a mixture of I_2 , THF (tetrahydrofuran), H_2O and
lutidine followed by hydrolysis of the triester to yield a
phosphate diester. The appropriate choice of either set of
25 conditions allows the placement of the phosphorothioate
diester in the desired position with respect to the native
phosphate diester. This technique allows for selective
reactivity at a specific nucleotidyl site, and avoids
nonspecific reaction with other functional groups available
30 in the nucleic acid.

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The complex formed is described below:

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3'-Nucleoside



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(Internucleotidic Phosphorothioate Diester)

The phosphorothioate diester can subsequently be alkylated with fluorescent molecules or other probes such as, for example, biotin. In this procedure, the complex which results is referred to as a "phosphorothioate triester" (which comprises an internucleotidic residue, a phosphorothioate diester and a detectable marker). The means by which this procedure occurs, e.g., alkylation, refers to the displacement of the functional group (such as the bromine in monobromobimane) and the formation of a sulfur-carbon bond between the fluorescent marker and the phosphorothioate diester.

For purposes of fluorescent labeling techniques herein contemplated, various fluorophores can be employed, for example, monobromobimane (MBB), bromomethylcoumarin, as well as a variety of chromophores carrying bromoacetamides, iodoacetamides, aziridinosulfonamides or γ -bromo- α,β -unsaturated carbonyls; monobromobimane is preferred.

One of the most surprising advantages of this invention is that the present methodology permits the introduction of fluorescent dyes or other probes in a "post-assay" procedure. By "post-assay" procedure is meant, generally, that the phosphorothioate diester-containing DNA is used in the assay of choice, for example, in polyacrylamide gel electrophoresis, and the fluorescent molecule or other marker or probe can be introduced at a later time, for example, while the nucleic acid is embedded

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1 in the polyacrylamide gel matrix. The assay procedures
contemplated by the present invention in this context
include, for example, gel electrophoresis, Southern
hybridization, and DNA sequencing techniques such as are
described by Sanger, et al. (Sanger, et al., Proc. Natl.
5 Acad. Sci., 74: 5436-5467, 1977).

Gel electrophoresis as used here is typically
performed by running DNA samples down specific lanes in a gel
(e.g., a polyacrylamide gel or agarose gel), under controlled
current and temperature conditions for a short period of
10 time. This procedure leaves the DNA embedded in the gel
matrix.

Southern hybridization involves the use of a
blotting membrane to remove the fractionated nucleic acid
from the gel and allows for hybridization of labeled probes
15 to the nucleic acid on the surface of the blotting membrane.
Radioisotopic labeling (^{32}P) has been commonly employed for
the detection of nucleic acids resolved by electrophoresis or
after hybridization techniques.

Sanger DNA sequencing (also known as "dideoxy
20 sequencing") has previously been done using ^{35}S labeling.
This typically involves two steps. The labeling reaction is
initiated after annealing of the primer to the template. A
low concentration of dTTP, dGTP, dCTP and α -[^{35}S]dATP is
employed in order to elongate the primer and incorporate some
25 radioisotope. The second step involves adding the
termination mixture, which is a higher concentration of all
four dNTP derivatives plus one of the dideoxy derivatives
(ddNTP).

Post-assay fluorescent labeling techniques as
30 described herein permit the introduction of multiple
fluorescent molecules or other appropriate markers into the

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1 nucleic acid, e.g., after electrophoresis and "post-assay"
labeling of detecting oligodeoxynucleotides and DNA fragments
can be detected on the basis of, e.g., fluorescence, with
high sensitivity.

5 Detection of fluorescent markers can be achieved by
use of e.g., a standard long-wavelength ultraviolet
transilluminator, to view the DNA in the gel.

The labeling procedure is particularly useful in
conventional enzymatic procedures for the sequencing of DNA.
Instead of radioisotopic labeling as described in the Sanger
10 sequencing technique the four dNTP α S derivatives used in the
sequencing reaction can be substituted such that the DNA
fragments produced will contain phosphorothioate diesters at
all internucleotidic positions which can allow multiple
labeling and ultimately allow reading of large and small DNA
15 fragments. The labeling procedure is also applicable to site
specific identification of nucleotides by introducing at
least one phosphorothioate diester selectively into an
internucleotidic residue or DNA fragment or oligodeoxy-
nucleotide, labeling said phosphorothioate diester with a
20 marker and detecting said marker.

The aforesdescribed labeling technique can also be
applicable to hybridization studies using, e.g.,
membrane-bound nucleic acids.

A fluorescently labeled cloned DNA probe can be
25 used to localize specific nucleic acid sequences in mixtures
of DNA restriction fragments fractionated by gel
electrophoresis. A replica of the gel is made by
transferring all of the fractionated DNA fragments to a sheet
of nitrocellulose paper or similar membrane (the "blotting
30 membrane") by diffusion or electrophoresis. The
hybridization probe can be labeled before or after the

1 hybridization assay occurs. The locations of the fragments
that hybridize to fluorescently labeled DNA probes are then
identified by their fluorescence. Similarly, nitrocellulose
paper replicas can be made of crowded colonies of bacteria
growing on an agar surface so that hybridization of the paper
5 with a specific labeled probe can be used to identify the few
cells carrying a newly cloned specific DNA fragment.

The labeling and detection techniques herein
discussed, can also surprisingly be easily employed in DNA
diagnostics and DNA therapy. The present advantage, relative
10 to art recognized techniques, is particularly manifest in
that the presence of the phosphorothioate diester does not
effectively alter the biophysical nature of the DNA and yet
selectively introduces a nucleophilic site which is readily
modified and exploited for diagnostic and therapeutic
15 purposes. For example, the phosphorothioate diester can be
introduced into the DNA and subsequently hybridized to a gene
of interest in vitro or in vivo, and then followed by
specific introduction of a probe to that gene. The probe to
the particular gene can then be used to discover the location
20 of the gene. This leads to detection of the presence or
absence of the gene under diagnostic investigation. The
probe can then be used in DNA therapeutics to inactivate or
destroy that particular gene or if necessary, to activate
that gene. For example, diagnosing genetic disorders and
25 direction of drug delivery (e.g., anticancer or antiviral
drugs).

Another surprising advantage of the present
invention is that the DNA-containing phosphorothioate diester
is largely resistant to nucleases and therefore is very
30 stable when introduced into complex biological systems found
in vitro and in vivo.

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1 The present invention can be used in spectroscopic
analysis (e.g., Nuclear Magnetic Resonance studies, and in
particular, the Nuclear Overhauser Enhancement [NOE]) to
measure distances within nucleic acids by use of probes which
can label specific phosphorothioate diesters.

5 The present invention can also be applied to
Electron Spin Resonance studies, which previously relied upon
the use of non-specific labeling. The simple and rapid
procedures described here will allow the preparation and
study of nucleic acid fragments containing spin labels,
10 attached at well-characterized locations. The procedure
described herein can also be used for the specific attachment
of hydrolytic reagents (e.g., ferric ion complexes),
intercalators and proteins to nucleic acids.

 Additionally, the present invention can also be
15 used to probe the structure of DNA fragments or
oligodeoxynucleotides by using chiral metal complexes (e.g.,
the Λ -isomer or Δ -isomer of tris-(4,7-diphenylphenan-
throlin) cobalt (III)) as the one marker of choice to be
attached to the phosphorothioate diester.

20 In order to use the phosphorothioate diester
effectively in a procedure for detecting nucleic acids, it is
advantageous to assess the stability, particularly with
respect to pH, of the labeled phosphorothioate diester-
fluorescent marker product. An HPLC analysis can be used
25 employing a reversed phase column. This assays the stability
of the labeled phosphorothioate derivative (triesters) over a
broad pH range during an incubation period at ambient
temperature.

 In another aspect of the present invention, high
30 detection sensitivity of fluorescent labeled nucleic acids
can be facilitated by the introduction of multiple

1 fluorescent markers to a corresponding multiple number of
phosphorothioate diesters earlier introduced at the selected
internucleotidic sites; the labeling reaction must occur at
adjacent phosphorothioate diesters such that, to achieve
maximum sensitivity, a nucleic acid fragment carries a
5 fluorophore at each and every internucleotidic phosphorus
residue. Surprisingly, experimentation indicates that there
is no steric hindrance or other difficulty in placing
fluorescent labels on adjacent phosphorothioate diesters,
thus permitting maximization of this technique.

10 As earlier discussed, "post-assay" labeling
procedures are useful for a variety of biochemical assays;
one of the most important specific applications involves the
detection of nucleic acids resolved by gel electrophoresis
techniques. One "post-assay" labeling procedure, for
15 example, can be accomplished using short oligodeoxynucleotide
fragments resolved by a given assay (e.g., gel
electrophoresis) and then soaking the gel containing the
small nucleic acid fragment with a solution which contains
the fluorescent marker of choice. Small fragments with
20 several labeled phosphorothioate diesters are quantitatively
compared with the fluorescence exhibited by a nucleic acid
fragment with a single fluorophore. There is a concomitant
increase in detection sensitivity with an increase in the
number of labeled phosphorus residues.

25 Longer DNA fragments containing phosphorothioate
diesters can be prepared by enzymatic synthesis when the
normal dNTP substrates are replaced by α -thio derivatives
(dNTP α S). In order to generate fragments of defined length,
an oligodeoxynucleotide primer can be extended using a
30 template (e.g., M13mp18 or M13mp19 or other single-stranded
DNA) and then the resulting material can be hydrolyzed with

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an appropriate restriction endonuclease. The amount of DNA
1 fragment which can be visualized is approximated based upon
the maximum amount of template present in the reaction
mixture or as the result of internal standardization via
radioisotopic labeling. The variety of bands produced can be
5 visualized by "post-assay" fluorescent labeling procedures.
The results show a further increase in sensitivity relative
to the increased sensitivity in small nucleic acid fragments.

Various fluorophores are available and many can be
employed in the present process. Any fluorophore can be
10 utilized for the "post-assay" fluorescent labeling procedures
contemplated by the present invention which reasonably
possess the following properties: high quantum yield;
solubility in aqueous (or largely aqueous) solutions;
relatively small size to allow diffusion through the gel
15 matrix; high fluorescence only after reaction with a sulfur
residue; and removal of the excitation maximum from the
absorbance maximum of the nucleic acids. One preferred
fluorophore which meets these criteria is monobromobimane.
Other fluorophores of choice can include, for example,
20 bromomethylcoumarin, or fluorophores carrying bromo- or
iodoacetamides, or aziridinosulfonamides. The fluorophores
of choice have the ability to alkylate the phosphorothioate
diester. The phosphorothioate diester is more nucleophilic
than any other site on the nucleic acid and results in
25 formation of a stable phosphorothioate triester when labeled
with the fluorophore of choice.

In particular, two widespread assays which can be
employed in conjunction with the "post-assay" fluorescent
labeling of this invention are DNA sequencing using, e.g.,
30 the Sanger dideoxy method and DNA hybridization (using e.g.,
the Southern technique).

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1. DNA Sequencing

1 Post-assay labeling is most amenable to enzymatic
dideoxy sequencing procedures. This approach incorporates
phosphorothioate diesters in place of native phosphate
diesters in the DNA fragments generated. After gel
5 electrophoresis, multiple fluorophores, such as MBB, can be
attached to the DNA via alkylation of the sulfur residue of
the phosphorothioate diesters.

 Current technology of Sanger sequencing utilizes
the dNTP derivatives. The Sanger sequencing technique
10 commonly utilizes a single α -[S³⁵]dNTP derivative to
introduce the radioactive label. However, by using all four
dNTP α S derivatives in the present invention, DNA fragments
can be generated by this technique which can contain hundreds
of phosphorothioate diesters. The "post-assay" labeling of
15 this invention can be directly applied to the detection of
these fragments.

 The "post-assay" fluorescent labeling technique
provides the sensitivity necessary to visualize DNA
sequencing ladders in the absence of radioisotopes. The
20 technique as described here employs all four dNTP α S
derivatives plus one of the dideoxy derivatives (ddNTP) in
the elongation and then termination of the DNA primer.
Sequencing ladders can be generated with dNTP α S substrates
in the like manner to the methodology with dNTP derivatives.

25 It is then desirable to vary the elongation and
termination conditions such that in the initial fluorescence
labeling the amount of DNA in each band may be varied. Then
the amount of DNA that appears in the bands can be maximized,
e.g., ranging from approximately 300 to 500 base pairs.
30 Fragments of this size can be resolved, and 300 to 500
fluorophores or other types of markers can be incorporated

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1 into such fragments. The distribution of the fragments can
be altered by changing the relative ratios of the
dideoxynucleotide/deoxynucleotides triphosphates.

5 A ddNTP/dNTP \propto S ratio of about 1:10 may be used to
obtain a distribution of small and large fragments. A
decrease in this ratio is effected to allow for more
efficient polymerization in a stepwise manner to as low as
about 1:500 in order to shift the distribution to longer
fragments.

10 The use of α -[³⁵S]dATP as a method for introducing
the radioisotopic label has been reported and is commonly
employed. Dideoxy sequencing using ³⁵S labeling typically
involves two steps. After annealing of the primer to the
template the labeling reaction is initiated. A low
concentration of dTTP, dGTP, dCTP and α -[³⁵S]dATP is
15 employed in order to elongate the primer and incorporate some
radioisotope. The second step involves adding the
termination mixture which is a higher concentration of all
four dNTP derivatives plus one of the dideoxy derivatives
(ddNTP). It is a simple procedure to then substitute the
20 four dNTP \propto S derivatives in both reactions (actually there is
only one reaction since no radioisotopic labeling is
involved) such that the DNA fragments produced will contain
phosphorothioate diesters at all internucleotidic positions.

25 For internal standardization, radioisotopic
labeling can be used in combination with fluorescent markers
to monitor the limits of detection sensitivity. To obtain
fragments which have been labeled to a known specific
activity a "minus-dCTP" labeling reaction is employed. This
uses a primer and template of known sequence, for example, of
30 the following sequences:

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1 M13mpl8 3'...CAAAGGGTCAGTGCTGCAACATTTTGCT...5'
primer 5'-GTTTTCCTCAGTCACGAC-3'

The labeling reaction can now be performed with low concentration of the dTTP α S, dGTP α S and α -[³⁵S]dATP. The elongation of the primer proceeds until the first dG present
5 in the template and then terminates resulting in the following sequence containing four ³⁵S labels:

M13mpl8 3'...CAAAGGGTCAGTGCTGCAACATTTTGCT...5'
elongated primer 5'-GTTTTCCTCAGTCACGACGTTCTAAAA-3'

10 The termination reaction uses all four dNTP α S derivatives at concentrations some two orders of magnitude higher than the labeling reaction such that any remaining radioactive α -[³⁵S]dATP is diluted and the quantity
15 available for incorporation becomes insignificant. The amount of material present (based upon the known specific activity of the α -[³⁵S]dATP) in a given band can now be easily determined by excising the band, lyophilizing the gel and determining the radioactivity present by scintillation
20 counting. By adjusting the concentrations of the template and primer as well as the ratio of the ddNTP to dNTP α S, the amount of DNA present in a given fragment can be altered. In addition, distribution of fragments can be shifted to those
25 of higher or lower molecular weight. Optimization of detection can allow "reading" of smaller fragments (smaller than 300 nucleotide residues). DNA sequencing in the
absence of radioisotopes can then be effectuated by detecting the hundreds of labeled, e.g., bi-manne-labeled phosphorothioate triesters by utilization of single or
30 sophisticated electronic techniques.

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2. DNA Hybridization

1 In another embodiment of the present invention, the
post-assay fluorescent labeling technique can also be applied
to hybridization studies using nucleic acids. The stability
of a native DNA duplex is first tested against nucleic acid
5 containing a number of phosphorothioate diesters and the
effect of this stability when the phosphorothioate diesters
are alkylated by a fluorophore is determined. For example,
the results for the detection of a 21-mer fragment containing
20 phosphorothioate diesters shows that in the absence of
10 electronic instrumentation it can readily be detected
visually. Nucleic acids with one label can be detected and
detection of single nucleotides can be facilitated. Such
visibility is increased proportionately with the proportionate
number of markers.

15 A 21-mer fragment is one example of a small
hybridization probe which can be used to detect nucleic acid
sequences. This is utilized in the following manner: DNA
fragments or oligodeoxynucleotides of reproducible size are
generated by selective chemical means, such as by a
20 restriction endonuclease enzyme. These nucleic acids are
resolved by a biochemical assay such as polyacrylamide or
agarose gel electrophoresis. The nucleic acid resolved in
this manner is then transferred to a blotting membrane, e.g.,
nitrocellulose membrane and the DNA probe is hybridized to
25 the nucleic acid. Although the DNA probe at this point has
the phosphorothioate diester or diesters incorporated into
it, the marker of choice, e.g., a fluorescent marker, may be
introduced before or after the hybridization assay.
Following these steps, the marker can be detected using
30 simple or sophisticated detection techniques.

One of the primary differences between "post-assay" fluorescent labeling within a gel matrix and labeling on a blotting membrane is that the latter occurs primarily on the surface of the membrane and not within a three dimensional matrix. With such surface phenomena it is possible to also use biotin labeled hybridization probes and detection with fluorescent protein complexes which could not be used for labels embedded in a gel matrix (the proteins involved are of large molecular weight and would not readily diffuse through the pores of the gel matrix). The phosphorothioate diester can be employed to allow efficient multiple (and specific) labeling with a biotin derivative. For example, the bromoacetamido group can be used to modify the phosphorothioate diester. A biotin derivative containing this functional group can be prepared quite simply by techniques available to one of ordinary skill in the art. Biotin labeling in this manner is considered an effective method for detecting nucleic acids when combined with immunochemical, histochemical or affinity detection systems. Two similar proteins, avidin and streptavidin, bind biotin very strongly and when coupled to fluorescent markers, enzymes or electron-dense proteins, can be exploited for the detection of nucleic acids. The use of fluorescent labeled antibodies raised against biotin can also be employed for detection. The biotin-labeled hybridization probe may be detected by use of a commercially available kit used for the detection of fluorescently labeled antibodies or by use of a transilluminator to detect the fluorescent group or protein.

Hybridization assays require the hybridization probe form stable Watson-Crick base pairs in order to localize the probe at a given sequence. The addition of biotin derivatives to the internucleotidic phosphorus

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1 residues can result in some destabilization of the double
stranded hybridization product. A series of biotin labeled
probes can be prepared containing from one to approximately
five biotin labels and the stability of the duplexes formed
can be examined with biotin modified oligodeoxynucleotides in
5 comparison with those unmodified. This can be accomplished
by labeling of the oligodeoxynucleotides containing the
correctly positioned (and number of) phosphorothioate
diester(s) and isolation of the product using HPLC
techniques. Duplex stability can be monitored by thermal
10 denaturation experiments and circular dichroism spectra.

The ability of the biotin labeled oligodeoxy-
nucleotide to function as a hybridization probe can then be
examined using, for example, the 21-mer previously described.
The sensitivity to detection of probes containing a varying
15 number of biotin labels can be examined using commercially
available fluorescent labeled proteins. "Spacing" the labels
every two, three or more phosphorus residues can be the
simplest route to enhance detection sensitivity.

In a second approach involving "post-assay"
20 labeling, the phosphorothioate-containing probe is hybridized
in one step; this avoids problems with the instability (if
any) of the biotin labeled hybridization product.
Subsequently, modification with the biotin label occurs, and
after removal of the excess label, the protein solution is
25 added for detection. This approach is conceptually similar
to the one described for the visualization of DNA sequencing
ladders and may also be the simplest approach to
hybridization assays.

Hybridization experiments can also be performed
30 with relatively long DNA fragments obtained from restriction
digests and multiple phosphorothioate diesters can be

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incorporated into such a fragment using DNA polymerase and
1 nick-translation procedures. Radioisotopic labeling is
accomplished by introducing "nicks" in the DNA with a dilute
solution of DNase I and then elongating the nicked sites
using DNA polymerase and the α -[³²P]dNTP substrates. The
5 radioisotopic derivatives can then be replaced with the
dNTP S derivatives and then hundreds of phosphorothioate
diesters can be incorporated into the fragment. The simplest
system to test hybridization can be one involving the M13 DNA
being used in the sequencing reactions. For example, M13 RF
10 (replicative form) DNA can be prepared in the conventional
manner and then cleaved out a 444-mer to use as a
hybridization probe. The 444-mer can then undergo
nick-translation to incorporate the phosphorothioate diesters
and then the modified and native sequences resolved by gel
15 electrophoresis. A second sample of the M13 RF DNA, for
example, can be digested such that the complementary 444-mer
restriction fragment (in addition to others) is produced and
transferred from an agarose gel to nitrocellulose or similar
blotting membrane. The hybridization can then proceed
20 followed by post-assay fluorescent labeling using, e.g.,
monobromobimane; fluorescent labeling with hundreds of
markers provides the desired detection sensitivity. Since
the monobromobimane is largely non-fluorescent until it
alkylates a sulfur containing functionality, the membrane
25 background fluorescence is relatively low. The labeled
marker can then be detected with relative ease.

In another embodiment of the present invention, DNA
probes are generated from mRNA. Again, one can simply use
the dNTP S derivatives, which function as substrates for
30 reverse transcriptase, to form the complementary DNA strand
for use as a hybridization probe. The use of the new

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1 labeling approach provides well-characterized hybridization
2 probes which can be used for the detection of specific DNA
3 sequences, in the absence of radioisotopes, for example, in
4 Southern blots, Northern blots, colony screening or plaque
5 screening.

5 3. Specific Modification of Nucleic Acids with
6 Fluorescent Markers or Spin Labels

7 In a further aspect of this invention, the labeling
8 of specific phosphorothioate diesters is also valuable for
9 structural studies involving fluorescent energy transfer
10 techniques and electron spin resonance (ESR) techniques.

11 The application of these two spectroscopic
12 techniques has long suffered from the difficulty in
13 specifically attaching the desired probe to the nucleic acid
14 fragment. The present procedure permits simple and rapid
15 synthesis of a variety of nucleic acid sequences which can be
16 easily modified with fluorescent markers or spin labels for
17 spectroscopic studies.

18 Fluorescent Energy Transfer Techniques allow for a
19 simple and rapid means for measurement of longer distances
20 within the nucleic acid structure, complementing NMR
21 techniques such as that of the Nuclear Overhauser Enhancement
22 (NOE) which can only measure small distances in the nucleic
23 acid.

24 The disadvantages of the energy transfer technique
25 have previously been in the difficulty of easily placing the
26 donor and acceptor chromophores in specific positions, and
27 the questionable accuracy of the technique when the
28 orientation of the chromophores is unknown.

29 These two shortfalls are eliminated by the labeling
30 of specific phosphorothioate diesters pursuant to the
31 methodology of the present invention. By controlling the

1 position of the phosphorothioate diester, the placement of a
specific label becomes as rapid as it is simple. Since the
label is oriented on the outer surface of the macromolecule a
freely rotating chromophore is likely.

5 ESR spectra can be valuable for the study of
biopolymer dynamics providing that the appropriate spin label
can be specifically bound to the macromolecule of interest.
In general, the technique has suffered a similar disadvantage
to energy transfer experiments in the difficulty of
specifically placing the label on the macromolecule. The use
10 of the phosphorothioate diester can again be valuable in this
respect. Nucleic acid fragments can be prepared with spin
labels by exactly the same approach as described above for
fluorescent markers. Specifically labeled probes can be
designed and prepared for these ESR studies.

15 Other procedures which can be used in association
with the instant technique involve optimization of
fluorescence detection. These include, for example,
1) altering the microenvironment of the labeled nucleic acid
fragments in the gel matrix to increase the quantum yield of
20 the fluorophore, 2) adjusting the excitation light energy to
optimally fit the excitation spectrum of the dye and using
filters to screen out all light energy (largely excitation
wavelengths) other than the desired emission energy, and
3) examining electronic detection as a means of automating
25 the reading of the information present. The first two
approaches together can be expected to increase the detection
sensitivity by roughly one order of magnitude. Electronic
methods can be expected to provide one or more additional
orders of magnitude enhancement.

30 The following examples would assist in further
detailing the subject invention herein.

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EXAMPLES

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1) Chemical Oligodeoxynucleotide Synthesis

5 Tp(s)T, the phosphorothioate diester derivative of TpT, is an example of the simplest phosphorothioate diester amenable to the labeling procedures described.

10 The (dT)₁₅ with phosphorothioate diesters 3' to thymidine residues 7, 8, and 9 were synthesized by using the phosphite triester methodology (Beaucage & Caruthers, Tetrahedron Lett., 22: 1859-1863, 1981) on a solid-phase CPG support. The synthesis was interrupted prior to the oxidation step when the incorporation of a phosphorothioate diester was desired. The normal oxidation step with 0.1 M I₂ in tetrahydrofuran/distilled water/lutidine (40:1:10) was replaced with a solution of 2.5 M sulfur in CS₂/lutidine
15 (1:1). The sulfur oxidation solution was injected directly onto the column with a syringe. After a reaction time of 1 h at ambient temperature, the column was washed with a 1:1 solution of CS₂ and lutidine to remove the residual sulfur. The column was then replaced on the machine, and the
20 synthesis cycle was resumed. The 21-mer d(GCTATCGAAAGATCTCATAAG) was synthesized in an analogous manner. The synthesis was interrupted at every oxidation step to allow oxidation with the sulfur solution.

25 Both oligodeoxynucleotides were deprotected in ammonia at 50°C for 18 h. Isolation was done by reverse-phase HPLC on a 9.4 x 250 mm column of MOS-Hypersil using a buffer of 50mM triethylammonium acetate, pH 7.0 with a gradient of 20-65% acetonitrile in 40 min.

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2) Solution Fluorescent Labeling Studies

1 The fluorophore of choice in this example, monobromobimane (MBB), was dissolved in acetonitrile, and stock solution (100mM) was stored in the dark at -20°C.

5 Typically, the oligodeoxynucleotides of interest were treated with an excess of monobromobimane, and the reaction was monitored by HPLC. Specifically, a solution of Tp(s)T (3.6 mM) in water was allowed to react overnight (18 h) with a 6-fold excess of monobromobimane (22 mM). The octamer (0.3 mM) in water was allowed to react with either a 10 5-fold excess of MBB (1.5 mM) or a 10-fold excess of MBB (3.0 mM). The fragment Tp(s)Tp(s)Tp(s)T (0.43 mM, a phosphorothioate diester concentration of 1.29 mM) was treated with an 8-fold excess (with respect to the phosphorothioate diesters) of MBB (10.5 mM). Covalent 15 fluorescent labeling of the 15-mer in solution (0.8 mM) with MBB was achieved at 7.5 mM MBB (3-fold excess for 2.4 mM phosphorothioate diester).

 The bimane-labeled Tp(s)T (see Figure 1) was isolated by reverse-phase HPLC on a 4.6 x 250 mm column of 20 ODS-Hypersil with 50 mM triethylammonium acetate, pH 7.0, and a gradient of 0-70% acetonitrile in 1 h. The other labeling reactions were monitored by reverse-phase HPLC on a 4.5 x 250 mm column of ODS-Hypersil with either 20 mM KH_2PO_4 , pH 5.5, and a gradient of 0-70% methanol in 30 min (the octamer and 25 tetramer) or 50 mM triethylammonium acetate, pH 7.0, and a gradient of 0-35% acetonitrile in 1 h (15-mer).

 Thin-layer chromatography studies were performed on silica gel thin-layer plates with a mobile phase of dichloromethane/methanol (9:1). 30

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3) pH Stability Studies

1 Duplicate reaction mixtures of 6 nmol of
bimane-labeled Tp(s)T were incubated at ambient temperature
in 50 mM buffer at the appropriate pH values. The following
5 buffers were used: pH values 3, 4 and 5, acetic
acid/potassium acetate; pH values 6 and 7, K_2PO_4/K_2HPO_4 ; pH
values 8 and 9, Tris-HCl; pH values 10 and 11, CAPS. At
various reaction times, the samples were analyzed by HPLC on
a 4.6 x 250 mm column of ODS-Hypersil using 0.02 M potassium
10 phosphate, pH 5.5, with a linear gradient of 0-70% methanol
in 30 min. The bimane-labeled Tp(s)T eluted at 21 min, while
the product TpT eluted at 16 min.

At low pH values (3-7) less than 5% of the triester
was hydrolyzed after a 20 h incubation as determined by
integration of the corresponding HPLC peaks. (see Figure 2).
15 Upon incubation with Tris-HCl at pH 8 for 20 h, 11% of the
triester was hydrolyzed. At pH 9, a 20 h incubation resulted
in 40% of the hydrolysis product. The triester was
completely hydrolyzed within 15 h at pH 10 and within 1 h at
pH 11 (see Figure 2). HPLC analysis confirmed that
20 hydrolysis occurred by cleavage of the P-S bond and formation
of TpT as expected.

To further characterize the reaction of
monobromobimane with a phosphorothioate diester, the reaction
was performed with an oligodeoxynucleotide which at ambient
25 temperature exists largely in the double-stranded form. The
reaction of the octamer d[GpCp(s)CpCpGpGpGpC] with a 10-fold
excess of monobromobimane was performed in either distilled
water or Tris-HCl pH 7, at ambient temperature. The HPLC
analysis after a 5-h incubation (Figure 3) showed the
30 starting material (14.88 min), a monobromobimane hydrolysis
product (15.3 min), a product peak (17.75 min), and

monobromobimane (25.21 min). The starting material was
1 completely consumed within 23 h. With a 5-fold excess of
monobromobimane, the reaction was complete within 48 h. The
reaction proceeded equally well with either the R_p or the S_p
diastereoisomer. A control reaction containing an
5 oligodeoxynucleotide with only phosphodiester failed to show
any conversion to a labeled product.

4) ^{31}P NMR Studies

The ^{31}P NMR studies were done at 121.5 MHz using a
varian multinuclear FT-NMR. Positive chemical shift values
10 are reported in parts per million (ppm) downfield from the
external standard of aqueous 85% phosphoric acid. NMR
analysis was done on a sample containing 1.2 μmol of
Tp(s)Tp(s)Tp(s)T (3.5 μmol of phosphorothioate diesters) and
20 mM Na_2EDTA . The sample was adjusted to a volume of 250 μL
15 with D_2O . After NMR analysis of the tetramer, 10 μmol of
monobromobimane (a 3-fold excess with respect to the
diesters) in 100 μL of acetonitrile was added to the NMR tube
with a final volume of 350 μL . The sample was allowed to
react for 2.5 h at ambient temperature in the dark. NMR
20 analysis was then repeated.

5) Radioisotopic Labeling (^{32}P End Labeling)

A reaction mixture in a final volume of 200 μL
containing 40.1 μM 15-mer (1 A_{260} unit), 40.7 μM ATP, 10 mM
MgCl₂, 10 mM dithiothreitol, 5 $\mu\text{g/mL}$ bovine serum albumin, 40
25 mM Tris-HCl, pH 8.7, 0.127 μM (0.152 mCi) [γ - ^{32}P]ATP, and 10
units of T₄ polynucleotide kinase was incubated at 37°C for
18 h. After the addition of the reaction mixture to the
Sep-pak cartridge (prewashed with 20 mL of methanol and 20 mL
of distilled water), it was washed with 10 mL of 1% aqueous
30 methanol to elute the unincorporated ATP and buffer salts.
The oligodeoxynucleotide was eluted with 10 mL of 50% aqueous

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methanol. The solution containing the DNA fragment was
1 evaporated to dryness and redissolved in 0.4 M distilled
water. Isolated yields ranged from 60 to 80%.

The 21-mer, 23.3 uM (1 A₂₆₀ unit), was end labeled
in an analogous manner but could not be eluted with aqueous
5 methanol. In this case, the Sep-pak cartridge was prewashed
with acetonitrile and distilled water. The unincorporated
ATP and salts were then eluted with 1% aqueous acetonitrile
while the oligodeoxynucleotide was eluted with 50% aqueous
acetonitrile. Isolated yields also ranged from 60 to 80%.

10 6) Post-Assay Labeling

Gel electrophoresis was performed on 20 x 20 x
0.04 cm or 34 x 42 x 0.04 cm gels of 20% acrylamide, 2%
bis(acrylamide) [or 6% acrylamide and 0.6% bis(acrylamide)],
50 mM Na₂EDTA, and 13 mM sodium persulfate. Post-assay
15 labeling was performed both in the presence and in the
absence of 7 M urea. The DNA was fixed in the gel by soaking
it in 10% aqueous acetic acid for 5 min. The gel was then
transferred to a 4 mM solution of monobromobimane in 50%
aqueous acetonitrile and allowed to react overnight (18 h) in
20 the dark. The gel was destained by shaking in 50% aqueous
acetonitrile for 1 h. The short destaining appeared
necessary because of minor reactions with the gel components
and monobromobimane. Following a brief treatment (5 min) in
60 or 75% aqueous dimethylformamide, the DNA was viewed on a
25 standard long-wavelength ultraviolet transilluminator (λ_{\max} =
366 nm). In some cases for internal standardization, the
fluorescent bands of DNA were cut out of the gel and
lyophilized before determination of the amount of DNA present
in the gel via scintillation counting.

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1 The effect of solvents on fluorescent intensity was
also investigated. After post-assay labeling and destaining,
the gels were treated with one of the following: 75% aqueous
mixtures of methanol, ethanol, butanol, dimethylformamide, or
concentrated glycerol. The gels were viewed using a long
5 ultraviolet wavelength light transilluminator.

7) Fluorescent Studies

 The fluorescence (excitation 385 nm, emission
465 nm) of varying solutions of bimane-labeled Tp(s)T in 5 mM
KH₂PO₄, pH 4.5, was measured by using a fluorescence
10 spectrophotometer, and a standard curve of fluorescence vs.
phosphorothioate diester concentration was fitted to the data
employing a linear least-squares analysis.

 After post-assay fluorescent labeling (see above)
with monobromobimane, the 5'-³²P end-labeled 15-mer was
15 electroeluted for 2 h from a 20% polyacrylamide gel into
dialysis tubing containing 0.5x TBE buffer. The solution was
evaporated to dryness, redissolved in 1 mL of distilled
water, and desalted using a column of Sephadex G-10. The DNA
fragment was collected, evaporated to dryness, and
20 redissolved in 3 mL of 5 mM KH₂PO₄, pH 4.5. The fluorescence
of the solution was measured and the concentration of the
15-mer determined by scintillation counting. The
fluorescence as a function of concentration of the
phosphorothioate diesters was plotted on the standard
25 bimane-labeled Tp(s)T curve.

 In similar fashion, the 5'-³²P end-labeled 21-mer
was electroeluted for 24 h from the polyacrylamide gel after
post-assay labeling. The solution was evaporated to dryness
and redissolved in 0.5 mL of distilled water. In this case,
30 the solution containing the 21-mer was adjusted to 10 mM
MgCl₂ and 2 M ammonium acetate, 1 volume of ice-cold

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1 acetonitrile was added, and the solution was kept at -70°C
for 18 h. The salt precipitated out of solution while
essentially all of the DNA remained in the supernatant. The
solubility of the labeled 21-mer in acetonitrile is largely a
5 result of the increased hydrophobicity conferred upon the
oligonucleotide due to the presence of the bimeane residues.
The supernatant was decanted, evaporated to dryness, and
dissolved in 3 mL of 5 mM KH_2PO_4 , pH 4.5. The fluorescence
and radioactivity were measured and compared with the
standard curve.

10 8) DNA Polymerase and Restriction Endonuclease Reactions

M13 mp18 DNA was converted to the replicative form
(RF) as follows. The template DNA (2.5 ug) and universal
primer (0.1 ug) were annealed in 25 uL of buffer containing
100 mM NaCl, 20 mM MgCl_2 , and 100 mM Tris-HCl, pH 8.0, by
15 heating the mixture to 56°C for 15 min followed by slow
cooling to ambient temperature. The final 50-uL reaction
mixture containing dATP, dGTP, dCTP, dTTP (500 uM each), ATP
(1 mM), DNA polymerase 1 (Escherichia coli, 10 units), and
T4 DNA ligase (8 units) was incubated overnight at 16°C .
20 Substitution of the appropriate dNTP α S derivative(s) for the
corresponding dNTP(s) essentially as described (Taylor,
et al., Nucleic Acids Res., 13: 8749-8764, 1985) allowed the
enzymatic incorporation of phosphorothioate diesters in place
of phosphodiester. In some cases for internal
25 standardization, α -[^{35}S]-dATP (1.15 Ci/mmol) was employed in
the elongation reaction.

Restriction digests with AvaI and HpaII were
performed as follows. The AvaI reaction mixture contained RF
M13mp19 DNA, 100 mM NaCl, 20 mM MgCl_2 , and 100 mM Tris-HCl,
30 pH 8.0. The HpaII reaction mixture contained RF M13mp18 DNA,
3 mM KCl, 5 mM MgCl_2 , 100 ug/mL BSA, and 5 mM Tris-HCl,

pH 7.4. The reactions were initiated by the addition of the
1 enzyme and incubated at 37°C for 2 h. The reaction mixture
was loaded onto 6% acrylamide, 0.6% bis(acrylamide) gels (20
x 20 x 0.04 cm or 34 x 42 x 0.04 cm) containing 3 mM Na₂EDTA,
7 M urea, and 50 mM Tris-borate, pH 8.3. Fluorescent
5 labeling proceeded as described above.

9) Detection of Nucleic Acids

The 5'-³²P end-labeled 21-mer was viewed on a
transilluminator ($\lambda_{\text{max}} = 366 \text{ nm}$) after gel analysis and
post-assay labeling. The bluish green bands were excised
10 from the gel and lyophilized, and the amount of DNA present
was determined by scintillation counting. The amount of the
oligodeoxynucleotide visible as a result of the bimane
fluorescence has decreased such that 500 fmol (500 x 10⁻¹⁵
mol) of the DNA fragments could be observed.

15 Longer DNA fragments containing phosphorothioates
can be prepared by enzymatic synthesis if the dNTP substrates
are substituted by the α -thio derivatives (Taylor et al.,
Nucleic Acids Res., 13: 8749-8764, 1985). In order to
generate fragments of defined length, an oligonucleotide
20 primer was extended using an M13mp18 or M13mp19 template and
the resulting material was hydrolyzed with a restriction
endonuclease. It was possible to prepare M13 RF DNA
containing phosphorothioates at each position. Cleavage of
the elongated DNA with HpaII produced fragments which
25 migrated in the 6% polyacrylamide gel and could be visualized
by post-assay fluorescent labeling (Figure 4A). A similar
experiment with the AvaI restriction endonuclease produced a
444-nucleotide fragment which could be visualized by
post-assay covalent labeling (Figure 4B). Some high
30 molecular weight DNA could also be observed in this gel at
the edge of the sample well (Figure 4B). With the 444-mer,

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1 the bands were excised, and the amount of DNA was determined
by scintillation counting. Approximately 40 fmol (40×10^{-15}
mol) of the 444-mer (containing a maximum of 104
bimane-labeled phosphorothioate diesters) could be visualized
in this experiment.

5 10) Synthesis of oligodeoxynucleotides containing
a single phosphorothioate diester

Two oligonucleotides were synthesized for covalent
attachment of a variety of reporter groups, including spin
labels, fluorophores and drug derivatives. A
10 dodecadeoxynucleotide and an eicosodeoxynucleotide were
chemically synthesized by the phosphoramidite method
described in Example 1 and altering the oxidation step at the
appropriate cycle, resulting in two phosphorus diastereomers
(Rp and Sp). It is possible to prepare the oligonucleotide
15 such that it contains a pure phosphorus diastereoisomer as
described [Connolly *et al.*, Biochemistry 23: 3443-3453,
1984; Taylor *et al.*, 1985].

Specifically, the dodecamer has the sequence
d[CGCA(s)AAAAAGCG] and the eicosomer has the sequence
20 d[CGTACTAGTT(s)AACTAGTACG].

Additionally Tp(s)T was reacted with a number of
fluorophores or reporter groups containing a variety of
functional groups. Three functionalities, γ -bromo- α,β -un-
saturated carbonyls, iodo (or bromo) acetamides, and
25 aziridinyI sulfonamides, were observed to effectively label
phosphorothioate diesters and produce the corresponding
phosphorothioate triester carrying the desired reporter
group.

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11) Phosphorothioate triester1 oligodeoxynucleotides carrying various reporter groups

Oligodeoxynucleotides of Example 10 containing a single covalently bound reporter group (Fig. 5) were obtained by incubation of the phosphorothioate-containing DNA fragment
5 with the reporter group of choice in aqueous or largely aqueous solutions at pH values from 5 to 8. These reactions were performed at 25 to 50°C and usually proceeded with yields greater than 85% after 24 h at 50°C. Resolution of
10 the reaction mixture and isolation of the triester product was accomplished by using HPLC (4.6 X 250 mm Hypersil-ODS with 0.02 M KH_2PO_4 pH 5.5 and a methanol gradient). Modification of the phosphorothioate was observed to be more efficient for the single-stranded dodecamer than the
15 self-complementary eicosomer. This difference in reactivity was partially overcome when the reaction mixture was heated at 50°C. In the absence of the phosphorothioate diester, control reactions using native oligodeoxynucleotides did not result in any significant labeling.

a) Attachment of a PROXYL spin label:

20 The reaction to produce the compound in Fig. 5a was conducted as described above using the following specific conditions: 10 mM 3-(2-iodoacetamido)PROXYL, 0.15 mM dodecamer, pH 8.0 (phosphate) at 50°C in a solution containing 4% DMP. Similar conditions were employed to label
25 the eicosomer.

b) Attachment of a CC-1065 drug analogue: a derivative of the dihydropyrroloindole subunit:

The reaction to produce the compound in Fig. 5b was conducted as described above using the following
30 specific conditions: 5 mM dihydropyrroloindole derivative,

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1 0.07 mM dodecamer, pH 8.0 (Tris) at 50°C in a solution
containing 60% DMF. This reaction required 48 h at 50°C or
80 h at 25°C at which time it was 70-80% complete. Similar
conditions were employed to label the eicosomer.

5 c) Attachment of a sulfonamide-linked dansyl
fluorophore:

The reaction to produce the compound in Fig.
5c was conducted as described above using the following
specific conditions: 12 mM N-dansylaziridine, 0.34 mM
dodecamer, pH 8.0 (phosphate) at 25°C in a solution
10 containing 50% acetonitrile. Similar conditions were
employed to label the eicosomer.

At 50°C, HPLC analysis of the dansylaziridine
reaction indicated the presence of minor products, suggesting
some nonspecific reaction with the DNA. Labeling conducted
15 at 25°C (pH 8.0) proceeded more slowly, but did not indicate
the presence of any species other than the desired product
and starting materials. However, the possibility of some
nonspecific modification of the DNA even at 25°C can not be
excluded.

20 d) Attachment of an N-linked dansyl fluorophore:

The reaction to produce the compound in Fig.
5d was conducted as described above using the following
specific conditions: 10 mM 1,5-I-AEDANS, 0.80 mM dodecamer,
pH 6.0 (phosphate) at 50°C in a solution containing 25% DMF.
25 Similar conditions were employed to label the eicosomer.

12) Stability and properties of phosphorothioate
triesters from examples 10 and 11

The unlabeled dodecamer helix, d[CGCA(s)AAAAAGCG]
d[CGCTTTTTTGCG], exhibited a T_m of 55°C, and this was
30 indistinguishable from the T_m values obtained for the PROXYL-
labeled (a in Figure 5) or drug-labeled (b in Figure 5)

1 helices. The T_m value for the self-complementary eicosomer,
d[CGTACTAGTT(s)AACTAGTACG]₂ with two labels was also largely
unchanged (68.5°C) in comparison to the unlabeled fragment
(T_m = 67°C).

5 The hydrolytic stability of the phosphorothioate
triesters is an important practical consideration for the
value of such derivatives in many studies. Hydrolysis of the
triesters proceeded by desulfurization (monitored by HPLC and
confirmed by comparison with authentic standards). No
10 detectable cleavage of the oligodeoxynucleotide at the point
of attachment was observed. This agrees with the results of
ethylated or hydroxyethylated derivatives, which result in
primarily desulfurization and only very minor amounts of
chain cleavage.

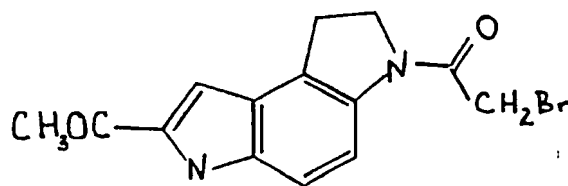
15 Less than 5% of the Tp(s)T triester carrying the
PROXYL spin label was hydrolyzed after 24 h at pH 7. At pH 8
this increased to 28%, and at pH 10 the triester was
completely hydrolyzed within 11 h. With longer fragments,
the hydrolytic stability of the triester increased [the
20 labeled dodecamer was hydrolyzed <1%, 30%, and 99% at pH
values 7, 8, and 10, respectively; the values for the
eicosomer were <1%, 2%, and 63%(24 h)]. The triester
prepared from a γ -bromo- α, β -unsaturated carbonyl (b in
Figure 5) exhibited stability similar to that of the
PROXYL-labeled derivatives while that resulting from reaction
25 with the aziridinyl sulfonamide (c in Figure 5) was more
stable [the Tp(s)T-labeled triester was hydrolyzed <1% (pH
7), 5% (pH 8), and 34% (pH 10) after 24 h at ambient
temperature].

30 It is noteworthy that the triester produced from
1,5-I-AEDANS and Tp(s)T was significantly less stable than
the PROXYL-labeled derivative although the triesters formed

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1 both resulted from iodoacetamides. The AEDANS-labeled dimer
exhibited 19% (pH 7) and 88% (pH 8) hydrolysis (24 h); it was
completely hydrolyzed within 2 h at pH 10. However, the
AEDANS-labeled dodecamer (d in Figure 5) exhibited only
1%, 49%, and 99% hydrolysis at the same respective pH values
5 (24 h).

An additional dodecamer was labeled with the
bromoacetamideo derivative i. Although the three
acetamido-linked adducts are similar in structure, that
prepared from i proved to be more stable than either a or d
10 (Figure 1) (only 13% of the triester formed from i was
hydrolyzed after 24 h at pH 8.0).
Derivative i:



WE CLAIM:

- 1 1. A nucleic acid detection product comprising a phosphorothioate diester covalently complexed with an internucleotidic residue wherein said phosphorothioate diester is also complexed with a detectable marker.
- 5 2. The product of Claim 1 wherein said marker is a fluorescent marker.
3. The product of Claim 2 wherein said fluorescent marker is monobromobimane.
4. The product of Claim 2 wherein said fluorescent
10 marker is bromomethylcoumarin.
5. The product of Claim 2 wherein said fluorescent marker carries a bromoacetamide, iodoacetamide, an aziridinosulfonamide, or a γ -bromo- α,β -unsaturated carbonyl group.
- 15 6. The product of Claim 1 wherein said marker is biotin, or a biotin derivative.
7. The product of Claim 1 wherein said marker is a spin label or spin probe.
8. The product of Claim 7 wherein said spin label
20 is PROXYL.
9. The product of Claim 1 wherein said marker is a metal complex.
10. The product of Claim 1 wherein said marker is a drug or a drug analog.
- 25 11. The product of Claim 10 wherein said drug analog is a dihydropyrroloindole subunit of CC-1065.
12. A method of labeling nucleic acids comprising reacting a nucleic acid having a phosphorothioate diester in at least one internucleotidic phosphorus residue, with a
30 marker to form an internucleotidic residue-phosphorothioate diester-marker complex.

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13. A method of detecting nucleic acids, comprising
1 introducing at least one phosphorothioate diester into a DNA
fragment or oligodeoxynucleotide, labeling said
phosphorothioate diester with a marker, and detecting the
marker in complex with the nucleic acid.

5 14. A method of identifying nucleotides comprising
site-selectively introducing at least one phosphorothioate
diester into at least one internucleotidic residue of a DNA
fragment or oligodeoxynucleotide, labeling said
phosphorothioate diester with a detectable marker, and
10 detecting said marker.

15. The method of Claim 12, 13 or 14 wherein said
marker is a fluorescent marker.

16. The method of Claim 15 wherein said fluorescent
marker is monobromobimane.

15 17. The method of Claim 15 wherein said fluorescent
marker is bromomethylcoumarin.

18. The method of Claim 15 wherein said fluorescent
marker carries a bromoacetamide, iodoacetamide, an
aziridinosulfonamide, or a γ -bromo- α,β -unsaturated
20 carbonyl group.

19. The method of Claim 12, 13 or 14 wherein said
marker is biotin or a biotin derivative.

20. The method of Claim 12, 13 or 14 wherein said
marker is a spin label or spin probe.

25 21. The method of Claim 20 wherein said spin label
is PROXYL.

22. The method of Claim 13 or 14 wherein said
marker is a metal complex.

23. The method of Claim 12 wherein said marker is a
30 drug or a drug analog.

- 1 24. The method of Claim 13 or 14, wherein said
labeling is conducted subsequent to resolution of the nucleic
acid by performance of a biochemical assay.
- 5 25. The method of Claim 13 or 14, wherein said
labeling is conducted prior to resolution of the nucleic acid
by a biochemical assay.
26. The method of Claim 24 or 25, wherein said
biochemical assay is gel electrophoresis.
- 10 27. The method of Claim 12, 13 or 14 wherein said
phosphorothioate diester is selectively introduced into a DNA
fragment or oligodeoxynucleotide at a specific nucleotidyl
site.
- 15 28. The method of Claim 27 wherein said
phosphorothioate diester is selectively introduced into said
DNA fragment or said oligodeoxynucleotide by an oxidation
reaction in the presence of elemental sulfur, CS₂ and
lutidine; and subsequently by a hydrolysis reaction in the
presence of a base.
- 20 29. The method of Claim 27 wherein said
phosphorothioate diester is introduced into said DNA
fragment or said oligodeoxynucleotide by chemical or
enzymatic techniques.
- 25 30. A method of DNA sequencing comprising:
a. introducing at least one phosphorothioate
diester into at least one selected site of a
DNA fragment or oligodeoxynucleotide by
enzymatic dideoxy sequencing procedures;
b. labeling each of said phosphorothioate diester
with a marker; and
c. detecting said DNA sequence.
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1 31. The method of Claim 30 which further comprises
generating said DNA fragment or oligodeoxynucleotide from
dNTP's derivatives and at least one of dideoxy derivative
(ddNTP).

5 32. The method of Claim 30, wherein said detection
procedure is automated.

 33. A method of DNA hybridization comprising:
 a. generating DNA fragments or
 oligodeoxynucleotides of reproducible size by
 selective chemical means;
10 b. resolving said DNA fragments or
 oligodeoxynucleotides by a biochemical assay;
 c. hybridizing said DNA fragments or
 oligodeoxynucleotides to a DNA hybridization
 probe having at least one internucleotidic
15 phosphorothioate diester;
 d. labeling said DNA hybridization probe with at
 least one detectable marker after said
 hybridization;
 e. detecting at least one marker in complex with
20 said hybridized probe.
 34. A method of DNA hybridization comprising:
 a. generating DNA fragments or
 oligodeoxynucleotides of reproducible size by
 selective chemical means;
25 b. resolving said DNA fragments or
 oligodeoxynucleotides by a biochemical assay;
 c. labeling a DNA hybridization probe having at
 least one internucleotidic phosphorothiate
 diester with at least one detectable marker
30 before hybridization with said DNA fragments or
 oligodeoxynucleotides;

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- 1 d. hybridizing said DNA fragments or
 oligodeoxynucleotides to said labeled DNA
 hybridization probe;
- e. detecting at least one marker in complex with
 said hybridized probe.
- 5 35. The method of Claim 33 or 34 wherein said DNA
 hybridization probe has an internucleotidic phosphorothioate
 diester at each internucleotidic phosphorus.
36. The method of Claim 33 or 34 wherein said
10 selective chemical means is digestion with restriction
 endonucleases.
37. The method of Claim 33 or 34 wherein said
 biochemical assay for resolution of said DNA fragments or
 oligodeoxynucleotides is polyacrylamide or agarose gel
 electrophoresis.
- 15 38. The method of Claim 33 or 34 wherein said DNA
 hybridization probe is prepared by the steps comprising:
- a. obtaining DNA fragments from restriction
 digests; and
- b. incorporating more than one phosphorothioate
20 diester into said DNA fragments by DNA
 polymerase and nick translation procedures.
39. The method of Claim 33 or 34 wherein said DNA
 hybridization probe is prepared from mRNA.
40. The method of Claim 39 wherein said
25 hybridization probe is prepared from mRNA by the steps
 comprising:
- a. providing dNTP α S derivatives to
 function as substrates for reverse
 transcriptase; and
- 30 b. synthesizing a complementary DNA strand to said
 mRNA by the action of reverse transcriptase,
 thereby forming said hybridization DNA probe.
- 35

41. The method of DNA hybridization according to
1 Claim 33 or 34 wherein said marker is a fluorescent marker.

42. The method of DNA hybridization according to
Claim 33 or 34 wherein said marker is monobromobimane.

43. The method of DNA hybridization according to
5 Claim 33 or 34 wherein said marker is bromomethylcoumarin.

44. The method of DNA hybridization according to
Claim 33 or 34 wherein said marker carries a bromoacetamide,
iodoacetamide, aziridinosulfonamide or γ -bromo- α, β -
unsaturated carbonyl group.

45. The method of DNA hybridization according to
10 Claim 33 or 34 wherein said marker is biotin or a biotin
derivative, and wherein the resulting product is a
biotin-labeled hybridization probe.

46. The method of Claim 45 wherein said detection
15 comprises:

- a. attaching a detectable protein to said
biotin-labeled hybridization probe; and
- b. identifying said probe.

47. The method of DNA hybridization according to
20 Claim 46 wherein said protein is avidin or streptavidin.

48. The method of DNA hybridization according to
Claim 46 wherein said protein is an antibody to said
biotin-labeled hybridization probe.

49. The method according to Claim 33 or 34 wherein
25 said DNA fragment or oligodeoxynucleotide is transferred to a
blotting membrane for the detection of specific DNA sequences
by Southern blots, Northern blots, colony screening or plaque
screening, thereby identifying DNA sequences under
investigation.

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- 1 50. A method for DNA detection comprising:
- 5 a. generating a DNA probe from DNA fragments or
 oligodeoxynucleotides having at least one
 phosphorothioate diester;
- b. hybridizing said DNA probe to a selected DNA
 sequence under investigation;
- c. labeling said probe with at least one
 detectable marker subsequent to said
 hybridization and thereby introducing said
 marker into the phosphorothioate diester; and
- 10 d. locating said selected DNA sequences under
 investigation by detecting said marker
 complexed with said hybridization probe.
51. A method for identifying DNA sequences
 comprising:
- 15 a. generating a DNA probe from DNA fragments or
 oligodeoxynucleotides having at least one
 phosphorothioate diester;
- b. hybridizing said DNA probe to a selected DNA
 sequence under investigation;
- 20 c. labeling said probe with at least one
 detectable marker subsequent to said
 hybridization and thereby introducing said
 marker into the phosphorothioate diester;
- d. locating said selected DNA sequences under
25 investigation by detecting said marker
 complexed with said hybridization probe; and
- e. directing drug delivery to said DNA sequence.
52. The method of Claim 51 which further comprises
 activating said DNA sequence.
- 30 53. The method of Claim 51 which further comprises
 inactivating said DNA sequence.
- 35

1 54. The method of Claim 51 which further comprises
degrading said DNA sequence.

55. A method of targeting a nucleic acid for
sequence-specific drug delivery which comprises:

- 5 a. preparing a sequence-specific nucleic acid
probe having at least one phosphorothioate
diester;
- b. labeling said phosphorothioate diester of said
probe with a drug or a drug analogue; and
- 10 c. hybridizing said probe with said nucleic acid
and thereby delivering said drug to a specific nucleic acid
target.

56. The method of Claim 55 wherein step b is
performed subsequent to step c.

15 57. The method of Claim 55 wherein said drug
analogue is a dihydropyrroloindole subunit of CC-1065.

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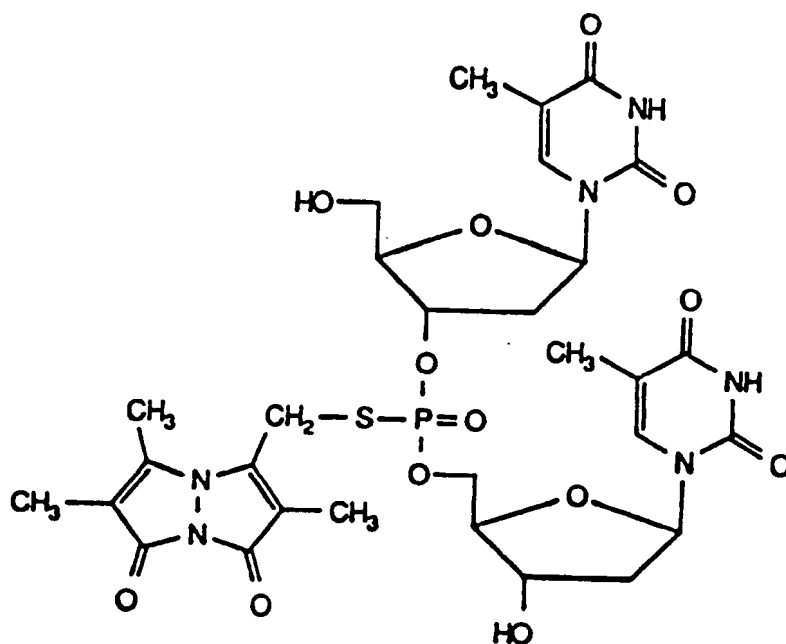


FIG. 1

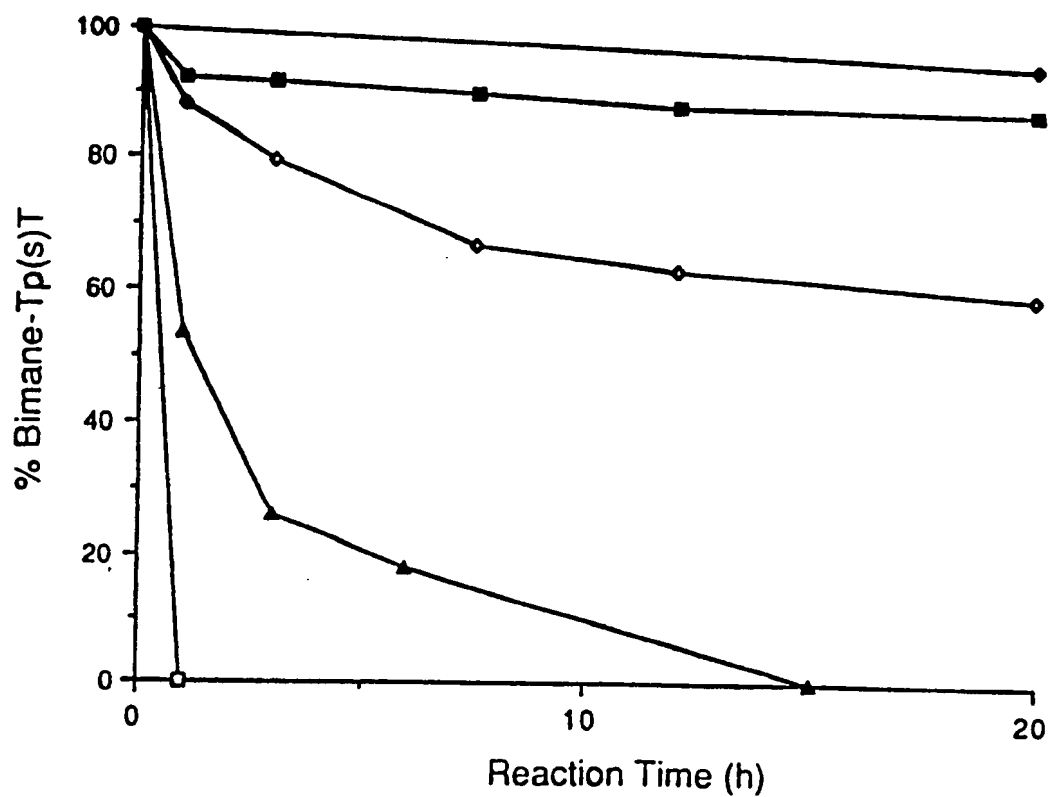


FIG. 2

SUBSTITUTE SHEET

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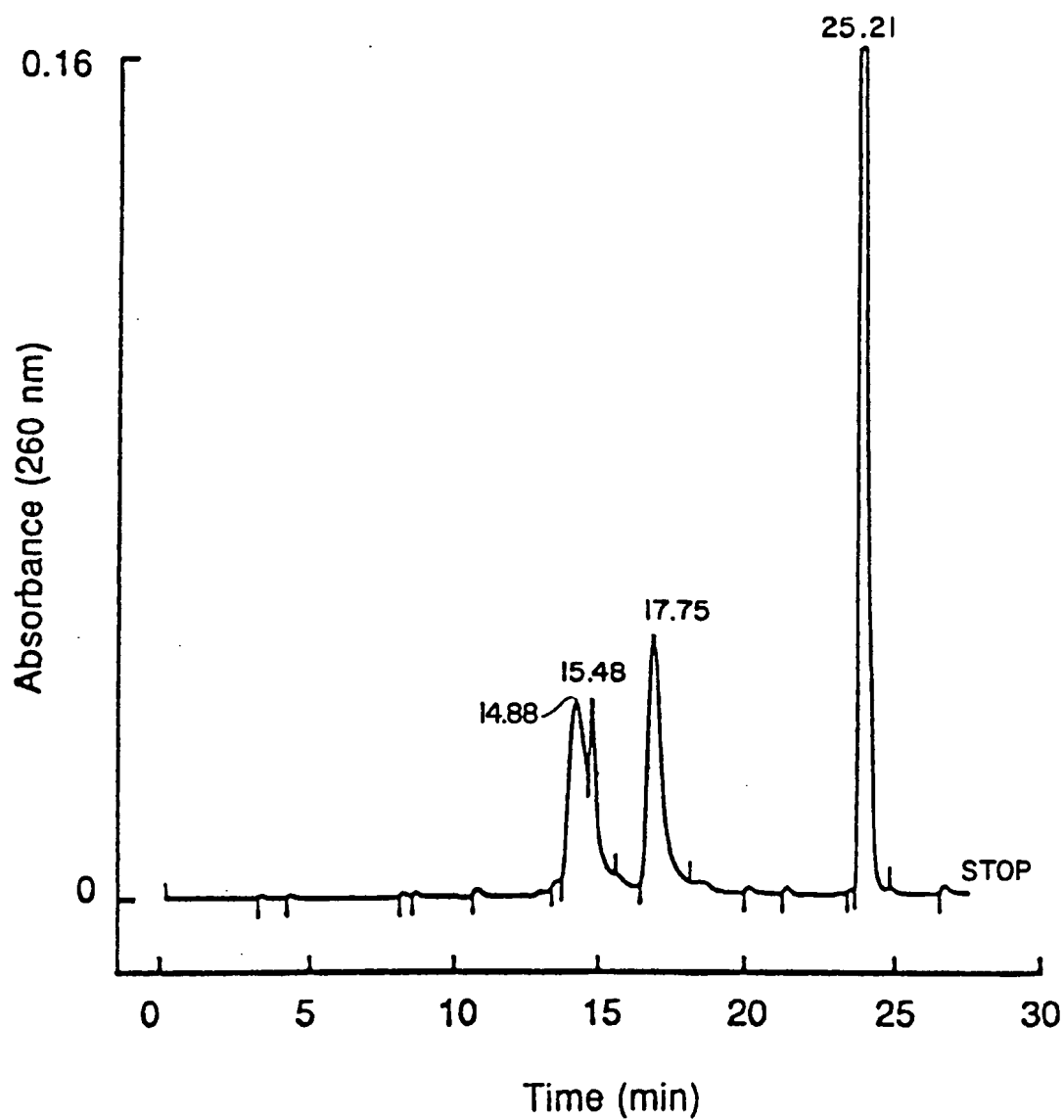


FIG. 3

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FIG.4A



SUBSTITUTE SHEET

FIG. 4B

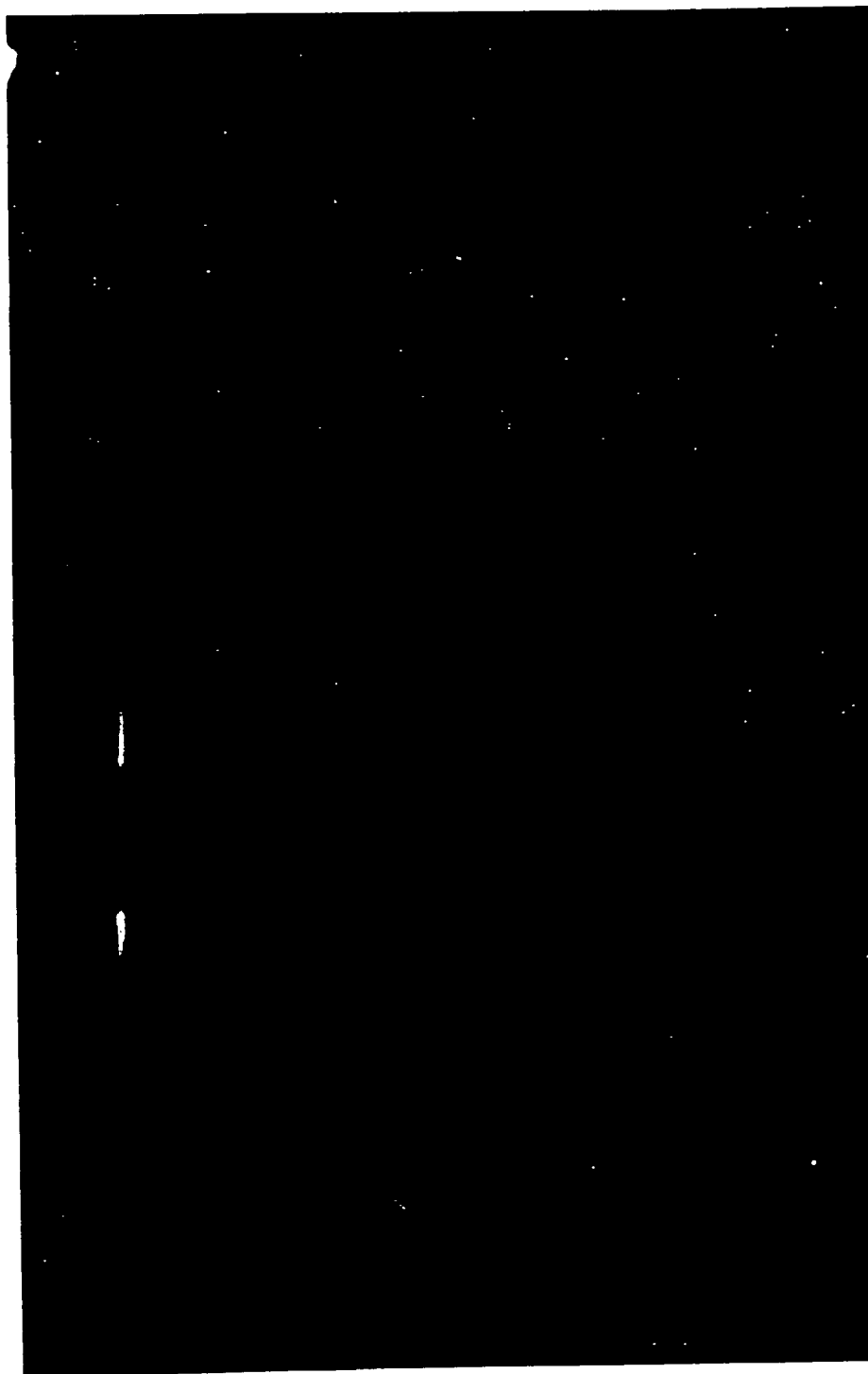
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SUBSTITUTE SHEET

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FIG. 5a

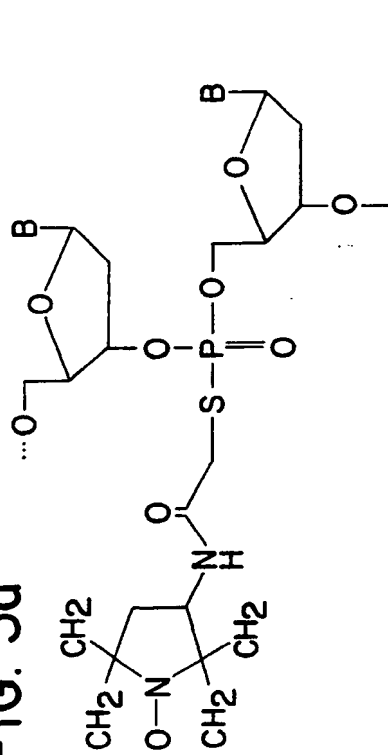


FIG. 5b

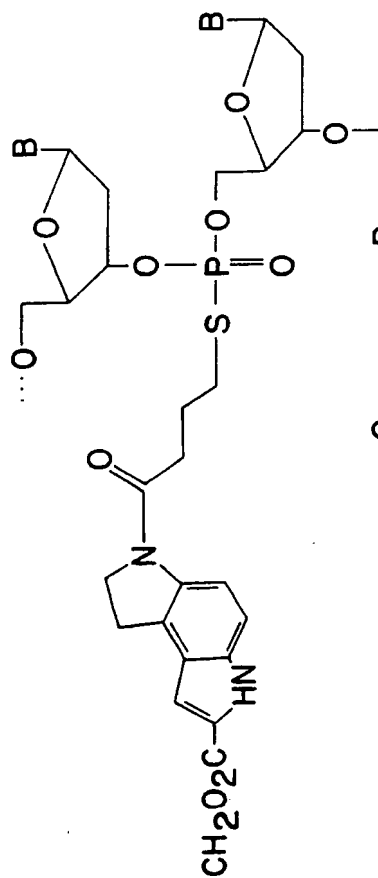


FIG. 5c

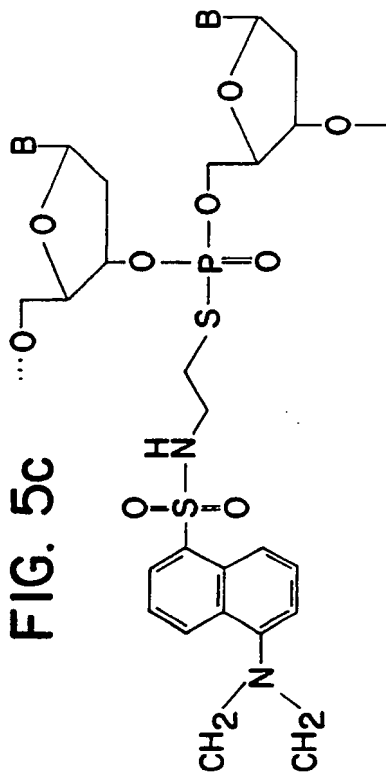
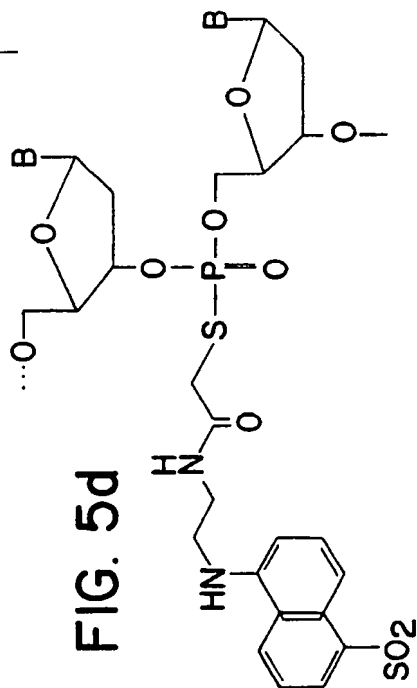


FIG. 5d



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/00182

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C12Q 1/68 U.S. CL.: 435/6; 536/27											
II. FIELDS SEARCHED <div style="text-align: center; margin-top: 10px;">Minimum Documentation Searched ⁷</div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%;">Classification System</th> <th style="width: 75%;">Classification Symbols</th> </tr> <tr> <td style="text-align: center; vertical-align: top;">U.S. CL.</td> <td style="text-align: center; vertical-align: top;">435/6, 436/800, 803, 804, 536/27, 935/86</td> </tr> </table> <div style="text-align: center; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div> <p style="text-align: center; margin-top: 10px;">Dialog Data Base: Biotech, APS</p>			Classification System	Classification Symbols	U.S. CL.	435/6, 436/800, 803, 804, 536/27, 935/86					
Classification System	Classification Symbols										
U.S. CL.	435/6, 436/800, 803, 804, 536/27, 935/86										
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category [*]</th> <th style="width: 60%;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 30%;">Relevant to Claim No. ¹³</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">US, A, 4,358,535, (FALKOW ET AL.) 09 November 1982 (See columns 4 and 5).</td> <td style="text-align: center; vertical-align: top;">1-29 & 50</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y,E</td> <td style="vertical-align: top;">US, A, 4,910,300, (URDEA ET AL.) 20 March 1990 (See examples 10-13 and the claims).</td> <td style="text-align: center; vertical-align: top;">1-29 & 50</td> </tr> </tbody> </table>			Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	Y	US, A, 4,358,535, (FALKOW ET AL.) 09 November 1982 (See columns 4 and 5).	1-29 & 50	Y,E	US, A, 4,910,300, (URDEA ET AL.) 20 March 1990 (See examples 10-13 and the claims).	1-29 & 50
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Y,E	US, A, 4,910,300, (URDEA ET AL.) 20 March 1990 (See examples 10-13 and the claims).	1-29 & 50									
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>											
IV. CERTIFICATION <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; vertical-align: top;"> Date of the Actual Completion of the International Search 02 MAY 1990 International Searching Authority ISA/US </td> <td style="width: 50%; vertical-align: top;"> Date of Mailing of this International Search Report <div style="text-align: center; font-size: 1.2em; font-weight: bold;">11 JUN 1990</div> <div style="text-align: center;"> Signature of Authorized Officer AMELIA BURGESS YARBROUGH </div> </td> </tr> </table>			Date of the Actual Completion of the International Search 02 MAY 1990 International Searching Authority ISA/US	Date of Mailing of this International Search Report <div style="text-align: center; font-size: 1.2em; font-weight: bold;">11 JUN 1990</div> <div style="text-align: center;"> Signature of Authorized Officer AMELIA BURGESS YARBROUGH </div>							
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